Examiner Russel objected that the paper sequence listing, submitted on August 1, 2001, did not include instructions to deleted the originally filed paper copy of the sequence listing and substitute the amended paper copy. The present response includes amendments correcting this oversight.

Examiner Russel objected that Applicant failed to expressly state that the sequence listing submitted on August 6, 2001 includes no new matter.

Responsive to Examiner Russel's objection, the undersigned hereby states that the content of the paper and computer readable copies of the Sequence Listing, submitted on August 1, 2001 in accordance with 37 CFR §1.821(a) through (c) and (e), respectively, are the same, except where noted, and include no new matter.

Conversion of Schemes into Figures:

Schemes 1-10 of the Specification are objected to under 37 CFR 1.58(a). Responsive to this objection, Applicant has deleted Schemes 1-10 from the Specification and resubmits the disclosure of schemes 1-10 herewith as Figures 1-10. The Figures 1-10 enclosed herewith are identical to Schemes 1-10 with respect to disclosure content. No new matter has been added.

A Brief Description of the Drawings has been added to the Specification in support of the newly inserted drawings. Support for the Brief Description of Drawings is found in the specification as follows:

Support for the description of Figure 1 is found in the Summary section on page 7, first paragraph.

Support for the description of Figure 2 of a general synthesis of Boc-amino thioester linker (**1**), dicyclohexylamine salt is found on pages 23-24.

Support for the description of Figure 3 of the progress of a first ligation reaction of the model peptide of Example 1 using a peptide fragment having a thioester derivative (Sequence No.: 1), as monitored by HPLC is found in Example 1, pages 27-29 (top).

Support for the description of Figure 4 is found in Example 1, page 29, second paragraph.

Support for the description of Figure 5 of the kinetics of the ligation reaction of Example 3 with respect to the linkage of peptide fragments IL-3 (46-76) and IL-3 77-95 fragments is found in Example 2, pages 31-32.

Support for the description of Figure 6 is found in Example 2, pages 31-32.

Support for the description of Figure 7 is found in Example 5, pages 38-40.

Support for the description of Figure 8 is found in Example 5, pages 38-40.

Support for the description of Figure 9 is found in Example 5, pages 38-40.

Support for the description of Figure 10 is found in Example 5, pages 38-40.

Abstract:

Examiner Russel objected that the application does not include an Abstract. The abstract from the priority International has been added by amendment to the present application.

A chemist of ordinary skill would readily appreciate that substitution of an extra cysteine into a naturally occuring protein for the purpose of employing the disclosed ligation process would be a preferred way of "expanding the scope of variation of the covalent structure of the protein molecule."

Claims 15-16, directed to sequence syntheses, have been cancelled without prejudice. Claims 15-16 are covered by Claim 8.

Rejection under 35 USC 112, second paragraph:

Claims 8-16, 19-23 and 27-31 have been rejected under Rejection under 35 USC 112, second paragraph as vague. Applicant's amendments obviate this basis for rejection.

Nonstatutory Double Patenting:

Claims 8-14 and 17-31 have been rejection on the basis of Nonstatutory Double Patenting. A terminal disclaimer has been submitted herewith.

Rejection under 35 USC 102(b):

Claims 8-10, 12-14, 17-19, 21-27 and 29-31 have been rejected as anticipated by WO Patent Application 96/34878 and by Dawson et al. Applicant's amendment adding a priority claim to WO Patent Application 96/34878 obviates this basis for rejection.

Rejection under 35 USC 102(b):

Claims 17-31 have been rejected as anticipated by references by Abrahmsen, Clark-Lewis, Bell, and Shaw. Applicant traverses this basis for rejection. Claims 17-23, 25, and 27-28 have been cancelled without prejudice. Claim 24 has been amended by

merger with claims 25 and 28 so as to obviate this rejection. More particularly, Claim 24, as amended, requires that:

(1) the first oligopeptide fragment has a length of 30 or more amino acid residues with a C-terminal non-β-branched amino acid residue modified as a C-terminal thioester;

(2) the synthetically produced protein be a derivative of a naturally isolatable protein or fragment thereof; and

(3) the *N*-terminal cysteine is not found in the naturally isolatable protein.

None of the cited prior art references discloses the above elements.

Support for "C-terminal non-β-branched amino acid residues modified as a C-terminal thioester" is found the specification as follows:

> "Furthermore, for optimal ligation, this component [i.e., the C-terminal residue] should have an unhindered (i.e. non β-branched) C-terminal amino acid." (Specification, page 14, bottom.)

Support for the "first oligopeptide fragment having a length of 30 or more amino acid" is found in Examples 2, 3, 4, and 5 of the Specification.

Support for the *N*-terminal cysteine is not found in the naturally isolatable protein is found in the Specification as follows:

> "The general synthetic access provided by the method of native chemical ligation greatly expands the scope of variation of the covalent structure of the protein molecule." (Specification, page 7, bottom of first paragraph)

None of the cited prior art references disclose the above elements. Accordingly, Claims 24, 26, and 29-31 are not anticipated by the cited prior art.

Summary:

Claims 9, 15-23, 25, and 27-28 have been cancelled without prejudice. Claims 8, 10-14, 24, 26 and 29-31 remain pending. Claims 8, 24, 30, and 31 have been amended. Claims 8, 10-14, 24, 26 and 29-31 are clear, fully supported by the specification, and unanticipated by the cited prior art. Allowance of Claims 8, 10-14, 24, 26 and 29-31 is respectfully requested.

Respectfully submitted,

6/21/02
Date

Donald G. Lewis, Reg. No. 28,636

THE SCRIPPS RESEARCH INSTITUTE
Office of Patent Counsel
10550 North Torrey Pines Road
Mail Drop TPC-8
La Jolla, California 92037
(858) 784-2937

[X] Attorney or agent of record
[ ] Filed Under §1.34(a)

APPENDIX

VERSION OF SPECIFICATION AND CLAIMS WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Page 1, prior to "Field of Invention" on line 1, please insert the following two new paragraphs:

Cross-Reference to Related Application:

The present application is a continuation application of and claims priority, under 35 U.S.C. § 120 , from US patent application Serial No. 08/710,653, which issued on February 6, 2001 as US Patent No. 6,184,344, and which was a national stage application under 35 U.S.C. § 371 of International Application No. PCT/US95/05668, filed May 4, 1995, which International Application was published in English.

Government Rights:

The invention disclosed herein was supported in part by Grants Number R01 GM 48897 and P01 GM 48870 from the National Institutes of Health. The United States government may have certain rights to this invention.

Page 1, lines 13-18, please delete the statement of Government Rights.

Page 7, first paragraph, lines 2-19, please substitute the following replacement paragraph:

One aspect of the invention is directed to a method of native chemical ligation. The method of native chemical ligation facilitates the chemical synthesis of proteins and large oligopeptide. The principle of

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`native chemical ligation` is shown in [Scheme 1] FIG. 1. The first step is the chemoselective reaction of an unprotected synthetic peptide-α-thioester with another unprotected peptide segment containing an N-terminal Cys residue, to give a thioester-linked intermediate as the initial covalent product. Without change in the reaction conditions, this intermediate undergoes spontaneous, rapid intramolecular reaction to form a native peptide bond at the ligation site. The target full length polypeptide product is obtained in the desired final form without further manipulation. The general synthetic access provided by the method of native chemical ligation greatly expands the scope of variation of the covalent structure of the protein molecule.

Page 9, last paragraph, line 28 to line 6 of page 10, please substitute the following replacement paragraph:

The oligopeptide thioester (α-COSR moiety) of FIG. 1 [Scheme 1] can be readily generated from a corresponding oligopeptide thiol (αCOSH) prepared by highly optimized stepwise SPPS on a thioester resin. The thioester resin was prepared by the method of L. E. Canne et al., Tetrahedron Letters (1995): vol. 36, pp. 1217-1220, incorporated herein by reference. The method of Canne employs the thioester resin disclosed by Blake and Yamashiro (J. Blake, Int. J. Pept. Protein Res. (1981): vol. 17, p 273; D. Yamashiro, et al., Int. J. Pept. Protein Res. (1988): vol. 31, p 322). Peptide products were cleaved, purified, and characterized by conventional methods. (M. Schnolzer, et al., Int. J. Pept. Protein Res.,(1992): vol. 40, pp 180-193.)

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Page 10, last paragraph, line 29 to line 14 of page 11, please substitute the following replacement paragraph:

The invention stated herein does not require the use of any protecting groups for the coupling of two oligopeptides because a less reactive (and thus more chemoselective) thioester electrophile is used instead of the acyl disulfide moiety (Yamashiro's approach). In the intermolecular coupling step, this thioester electrophile requires a more nucleophilic sulfhydryl moiety rather than a free amine. The nucleophilic sulfhydryl moiety can be found on cysteine residues. Since the amino and hydroxyl functionalities are relatively unreactive to the thioester electrophile, a selective coupling of the two unprotected oligopeptides is achieved with the cysteine sulfhydryl moiety. The sulfhydryl group on the cysteine of peptide 2 will first attack the thioester of peptide 1 and form a coupled thioester intermediate. This coupled thioester intermediate is concomitantly attacked by the free α-amino moiety from the cysteine and spontaneously rearranges to form the native peptide bond. Yields are therefore increased by eliminating protection and deprotection steps, since side undesired reactions are reduced (FIG. 1) [Scheme 1].

Page 11, last paragraph, line 35 to line 9 of page 12, please substitute the following replacement paragraph:

In this method (FIG. 2 [Scheme 2]), thiol 3 is generated from the reaction of chloride 2 (Yamashiro et. al. Int. J. Pept. Protein Res. (1988): vol. 31, p 322) with thiourea, followed by hydrolysis of the resulting thiouronium salt in aqueous base. Thiol 3 is a general intermediate which

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can be reacted with a wide range of commercially available Boc-amino acid succinimide esters to produce the desired thioester linker 1 which is conveniently isolated as the dicyclohexylamine (DCHA) salt.

Page 12, please delete the second paragraph starting on line 10 and extending to line 9 on page 14 and substitute the following amended paragraph:

Model studies were undertaken with small peptides to investigate the native chemical ligation approach. To help explore the mechanism of the reaction, the peptide Leu-Tyr-Arg-Ala-Gly-α-COSBzl (SEQ ID NO 3) was reacted with Ac-Cys. The exact mass of the resulting ligation product was determined by electrospray mass spectrometry, and was consistent with a thioester-linked peptide as the ligation product generated by nucleophilic attack of the Ac-Cys side chain on the α-thioester moiety of the peptide. Reaction of Leu-Tyr-Arg-Ala-Gly-α-COSBzl (SEQ ID NO 3) with H-Cys-Arg-Ala-Glu-Tyr-Ser (SEQ ID NO 2) (containing an unblocked α-NH_2 functional group) proceeded rapidly at pH 6.8 (below pH 6 the reaction proceeded very slowly, suggesting the involvement of the ionized thiolate form of the Cys side chain), and gave a single product of the expected mass. This product lacked susceptibility to nucleophiles, and had the ability to form disulfide-linked dimeric peptides, indicating unambiguously the formation of a native amide bond at the ligation site. These studies were consistent with the mechanism shown in FIG 1 [Scheme 1], in which the initial thioester ligation product was not observed as a discrete intermediate because of the rapid rearrangement to form a stable peptide bond. Facile intramolecular reaction results from the favorable geometric arrangement of the α-NH_2 moiety with respect to the

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thioester formed in the initial chemoselective ligation reaction. Use of such `entropy activation` for peptide bond formation is based on principles enunciated by Brenner. (M. Brenner, in Peptides. Proceedings of the Eighth European Peptide Symposium H. C. Beyerman, Eds. (North Holland, Amsterdam, 1967) pp. 1-7.) The concept of `entropy activation` for peptide bond formation has been more recently adopted by D. S. Kemp et al. (J. Org. Chem. (1993): vol. 58, p 2216) and by C.-F. Liu, et al. (J. Am. Chem. Soc. (1994): vol. 116, p 4149).

Page 16, please delete the second paragraph starting on line 10 and extending to line 9 on page 14 and substitute the following amended paragraph:

Further model reactions demonstrate that the use of better thioester leaving groups results in faster ligation reactions. We applied this observation to the native chemical ligation of peptides from the extracellular domain of a human cytokine receptor (R. D'Andrea, et al., Blood, (1994): vol. 83, p 2802.) as shown in FIG. 5 [Scheme 5]. Use of the 5-thio-2-nitrobenzoic acid (-SNB) leaving group, corresponding to the reduced form of Elman's reagent, gave rapid high yield reaction. As described below in connection with FIG. 5 [Scheme 5], the reaction between the peptide segments was observed to have gone essentially to completion in less than 5 minutes, giving the 50 residue product with a native peptide bond at the site of ligation. Thus, rapid native chemical ligation can be achieved by use of a thioester leaving group with suitably tuned properties.

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Page 16, please delete the last paragraph starting on line 19 and extending to line 18 on page 18 and substitute the following amended paragraph:

Application of the native chemical ligation method to the total synthesis of a protein molecule was illustrated by the preparation of human interleukin 8 (IL-8). (M. Baggiolini, et al., FEBS Lett. (1989): vol. 307, p 97; I. Clark-Lewis, et al., J. Biol. Chem. (1994): vol. 269, p 16075 (1994); I. Clark-Lewis, Biochemistry (1991): vol. 30, p 3128; and K. Rajarathnam, et al., (1994): Biochemistry, (1994): vol. 29, p 1689.) The 72 amino acid polypeptide chain contains four Cys residues, which form two functionally critical disulfide bridges in the native protein molecule. The total synthesis of IL-8 is shown in FIG. 7 [Scheme 7]. The two unprotected synthetic peptide segments reacted cleanly to give the full length polypeptide chain in reduced form without further chemical manipulation (9). This successful ligation was particularly significant because the 33- and 39-residue IL-8 segments each contained two Cys residues, and together encompassed 18 of the 20 genetically encoded amino acids found in proteins. The purified product was folded and oxidized as previously described, to give IL-8 with a mass precisely 4 daltons less than that of the original ligation product, indicating the formation of two disulfide bonds. The properties of this folded product were identical to those of previously studied authentic IL-8 samples. Titration in an assay for neutrophil elastase release demonstrated that the potencies (ED50=0.3nM) and maximal responses of the folded, ligated [Ala33]IL-8 and the corresponding molecule obtained by conventional synthesis were indistinguishable and identical to native sequence IL-8. This result unambiguously confirmed the formation of a peptide bond at the ligation

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site, because the thioester-to-amide rearrangement must have taken place to give the free Cys^{34} side chain that formed the native disulfide bond (see FIG. 7 [Scheme 7]).

Page 20, please delete the second paragraph, lines 6-12, and substitute the following amended paragraph:

The conditions stated above, permit the formation of an unprotected oligonucleotide which is equipped with the activated thioester. Subsequent reaction with a second peptide containing a terminal cysteine residue, permits a facile coupling with the formation of a native peptide bond and can generate oligopeptide chains of 100 or more amino acid residues (FIG. 1 [Scheme 1]).

Page 21, line 26 after last paragraph, please insert the following ten paragraphs directed to the Brief Description of Drawings Section, viz.:

Brief Description of Drawings:

Figure 1 illustrates the overall principle of 'native chemical ligation.'

Figure 2 illustrates a general synthesis of Boc-amino thioester linker (**1**), dicyclohexylamine salt.

Figure 3 illustrates the progress of a first native chemical ligation reaction for the production of model peptide (Sequence No. 5) of Example 1 using a first peptide segment (Sequence No. 2) and a second peptide seguement (Sequence No. 3) having a S-benzyl derivative, as monitored

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by HPLC.

Figure 4 illustrates the progress of a second native chemical ligation reaction for the production of model peptide (Sequence No. 5) of Example 1 using a first peptide segment (Sequence No. 2) and a second peptide seguement (Sequence No. 6) having a thioacetic acid derivative, as monitored by HPLC.

Figure 5 illustrates the kinetics of the native chemical ligation reaction of Example 2 with respect to the linkage of peptide segments 46-76 and 77-95 for producing IL-3 receptor β-subunit.

Figure 6 illustrates the reversed-phase HPLC purification and electrospray mass spectroscopy of unreacted IL-3 peptide segments (46-76) and (77-95) of IL-3 receptor β-subunit and of their native chemical ligation product as detailed in Example 2.

Figure 7 illustrates a native chemical ligation scheme for producing LI-8 as detailed in Example 3.

Figure 8 illustrates the reversed-phase HPLC purification and electrospray mass spectroscopy of IL-8 (Sequence No.: 10) as formed by a native chemical ligation of peptides Sequence No.'s 8 and 9 in Example 3. Figure 8B illustrates the unreacted IL-8 peptides (1-33)-SBenzyl (Sequence No. 8) and IL-8 34-72 (Sequence No. 9) prior to ligation; Figure 8C illustrates the unfolded IL-8 ligation product, Sequence No. 10; and Figure 8D illustrates the folded IL-8 ligation product, Sequence No.

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10.

Figure 9 illustrates the reversed-phase HPLC purification and electrospray mass spectroscopy of HIV-1 K41 protease (Sequence No.: 15) as formed by native chemical ligation of peptides Sequence No.'s 11 and 12 in Example 4.

Figure 10 illustrates the reversed-phase HPLC purification of Barnase K39 (Sequence No.: 20) as formed by native chemical ligation of peptide Sequence No's. 17 and 18 in Example 5.

Page 29, please delete the first full paragraph, lines 2-14, and substitute the following amended paragraph:

Another, unpublished model using the 2-thioacetic acid derivative Leu-Tyr-Arg-Ala-Gly-SCH_2COOH (SEQ ID NO 6), formed from attack of the thioacid Leu-Tyr-Arg-Ala-Gly-SH (SEQ ID NO 1), onto 2-bromoacetic acid in methylene chloride) + Cys-Arg-Ala-Glu-Tyr-Ser (Sequence No.: 2) was ligated at pH 6.8 in 0.2 M phosphate buffer, at 45 °C. After 1.0 hour the reaction had proceeded to 80% as observed in [Scheme] FIG. 4 by HPLC. The isolation of oxidation products from the ligated Leu-Tyr-Arg-Ala-Gly-Cys-Arg-Ala-Glu-Tyr-Ser (SEQ ID NO 5) and unreacted Cys-Arg-Ala-Glu-Tyr-Ser (SEQ ID NO 2) demonstrated the presence of a free thiol ligation product.

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In the Claims:

Please amend Claim 8 as follows:

8. (once amended) A method for producing a desired protein or domain thereof, which comprises admixing:

(I) a first oligopeptide, said first oligopeptide comprising a fragment of said desired protein or domain thereof, and having a C-terminal thioester; and

(II) a second oligopeptide, said second oligopeptide comprising a fragment of said desired protein or domain thereof, and having an N-terminal cysteine amino acid residue having an unoxidized sulfhydryl side chain and a free amino group that is capable of forming a β-aminothioester linkage with said C-terminal thioester that rearranges to form an amide bond therein between;

wherein said admixing is conducted under conditions sufficient to permit the formation of an amide bond between the C-terminus of said first oligopeptide [fragment] and the N-terminus of said second oligopeptide [fragment].

Please cancel claims 9, 15-16, and 17-23, without prejudice.

Please amend claim 24 as follows:

24. (once amended) A synthetically produced protein of greater than about 35 amino acid residues, wherein all of the residues of said protein are linked to adjacent residues

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via an amide bond, said protein being produced by the process of ligating together at least two oligopeptide fragments wherein:

(1) said first oligopeptide fragment [has] having a length of 30 or more amino acid residues with a C-terminal non-β-branched amino acid residue modified as a C-terminal thioester; and

(2) said second oligopeptide fragment has an *N*-terminal [amino acid residue] cysteine having an unoxidized sulfhydryl side chain and a free amino group that is capable of forming a β-aminothioester linkage with said C-terminal thioester that rearranges to form an amide bond therein between; wherein said ligation results in the formation of an amide bond linking said first and second fragments, wherein said synthetically produced protein being a derivative of a naturally isolatable protein or fragment thereof, said *N*-terminal cysteine not being found in the naturally isolatable protein.

Please cancel claims 25 and 27-28, without prejudice.

30. (once amended) The [method] synthetically produced protein of claim 29, wherein said mammalian protein is a human protein.

31. (once amended) The [method] synthetically produced protein of claim 30, wherein said human protein is a cytokine.

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In the Abstract:

After the Claims, please insert the following paragraph:

Abstract

Proteins of moderate size having native peptide backbones are produced by a method of native chemical ligation. Native chemical ligation employs a chemoselective reaction of two unprotected peptide segments to produce a transient thioester-linked intermediate. The transient thioester-linked intermediate then spontaneously undergoes a rearrangement to provide the full length ligation product having a native peptide bond at the ligation site. Full length ligation products are chemically identical to proteins produced by cell free synthesis. Full length ligation products may be refolded and/or oxidized, as allowed, to form native disulfide-containing protein molecules. The technique of native chemical ligation is employable for chemically synthesizing full length proteins.